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Cathelicidin Family of Antibacterial Peptides CAP18 and CAP11 Inhibit the Expression of TNF-α by Blocking the Binding of LPS to CD14$^+$ Cells[1]

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Mammalian myeloid and epithelial cells express several kinds of antibacterial peptides (α-/β-defensins and cathelicidins) that contribute to the innate host defense by killing invading micro-organisms. In this study we evaluated the LPS-neutralizing activities of cathelicidin peptides human CAP18 (cationic antibacterial proteins of 18 kDa) and guinea pig CAP11 using the CD14$^+$ murine macrophage cell line RAW264.7 and the murine endotoxin shock model. Flow cytometric analysis revealed that CAP18 and CAP11 inhibited the binding of FITC-conjugated LPS to RAW264.7 cells. Likewise, Northern and Western blot analyses indicated that CAP18 and CAP11 suppressed LPS-induced TNF-α mRNA and protein expression by RAW264.7 cells. Interestingly, CAP18 and CAP11 possessed LPS-binding activities, and they strongly suppressed the interaction of LPS with LPS binding protein that mediates the transport of LPS to CD14 to facilitate the activation of CD14$^+$ cells by LPS. Moreover, when CAP18 and CAP11 were preincubated with RAW264.7 cells, they bound to the cell surface CD14 and inhibited the binding of FITC-LPS to the cells. Furthermore, in the murine endotoxin shock model, CAP18 or CAP11 administration inhibited the binding of LPS to CD14$^+$ cells (peritoneal macrophages) and suppressed LPS-induced TNF-α expression by these cells. Together these observations indicate that cathelicidin peptides CAP18 and CAP11 probably exert protective actions against endotoxin shock by blocking the binding of LPS to CD14$^+$ cells, thereby suppressing the production of cytokines by these cells via their potent binding activities for LPS and CD14. *The Journal of Immunology,* **2001, 167: 3329–3338.**

Lipopolysaccharide is a major constituent of the outer membrane of Gram-negative bacteria and is recognized as a key molecule in the pathogenesis of Gram-negative sepsis and septic shock. LPS activates mononuclear phagocytes (monocytes and macrophages) and other types of cells to secrete TNF-α, IL-1β, IL-6, and other cytokines, which mediate the development of septic shock (1). In certain animal models TNF-α is principal for the lethality induced by LPS (2–4). Recent research has revealed the detailed mechanisms by which LPS activates mononuclear phagocytes (5–8). On release, LPS interacts with the LPS-binding protein (LBP),[3] an acute phase reactant that is present in the blood and transfers LPS to CD14, the primary receptor for LPS that exists as a soluble form in blood and as a GPI-linked molecule on the surface of mononuclear phagocytes (9–11). LPS-CD14 complexes initiate the intracellular signaling by binding to the recently defined membrane proteins Toll-like receptors (TLRs), which are structurally related to the *Drosophila* protein Toll and are expressed on mononuclear phagocytic cells and other cells (5–8). LPS-CD14 complexes activate NF-κB transcription factor as well as extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase, which mediate the production of inflammatory cytokines (5–8).

In mammals a number of cationic antibacterial peptides, such as defensins, are found in blood, secretions, epithelial tissues, and neutrophil granules (12–16). They are evolutionary ancient components that can kill the invading micro-organisms by perturbing their membranes and contribute to the innate host defense. Among these peptides, cathelicidin is a novel family of antibacterial peptides that have been isolated from epithelial tissues and myeloid cells of human and animal species (17). Precursors of cathelicidins are characterized by the highly conserved cathelin-like pro-sequences and variable carboxyl-terminal sequences that correspond to the mature antibacterial peptides. We have isolated two members of cathelicidins, CAP18 (cationic antibacterial proteins of 18 kDa) and CAP11 (cationic antibacterial polypeptide of 11 kDa) from rabbit and human neutrophils (18, 19), and guinea pig neutrophils (20, 21), respectively. CAP18 is a precursor form of cathelicidins, and its carboxyl-terminal antibacterial peptide comprising 37 aa residues (L^{1}LGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES^{37}) has been recently identified in humans (13). In contrast, CAP11 is a carboxyl-terminal antibacterial peptide cleaved from its precursor and is a homodimer of G^{1}LRKKFRKTR KRIQKLGRKIGKTGRKVWKAWREYGQIPYPCRI^{43}) joined with one disulfide bond (20, 21). CAP18 and CAP11 peptides exhibit potent antibacterial activities against both Gram-negative

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; CAP18, cationic antibacterial protein of 18 kDa; CAP11, cationic antibacterial polypeptide of 11 kDa; TLR, Toll-like receptor; TMB, 3,3′,5,5′-tetramethyl-benzidine; hBD, human β-defensin; MAC, minimum agglutinating concentration; MIC, minimum inhibitory concentration.

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and Gram-positive bacteria (18–21). Moreover, it has been found that a CAP18-derived peptide can block LPS-induced TNF-α release and reduce the mortality associated with endotoxemia in the galactosamine-sensitized murine model (22). Thus, derivatives of CAP18 could have therapeutic potential for Gram-negative bacterial sepsis and septic shock (23). However, little is known about the mechanisms by which CAP18-derived peptides block the biological activities of LPS. Moreover, it is unclear whether CAP11, a homologue of CAP18, also has LPS-neutralizing activity.

In this study, therefore, to elucidate the mechanisms of the protective actions of CAP18 and the actions of CAP11 on LPS, we investigated the effects of human CAP18 and guinea pig CAP11 peptides on the binding of LPS to CD14$^+$ cells and TFN-α expression by these cells using the murine macrophage cell line RAW264.7 and the murine endotoxin shock model. The results obtained indicated that CAP18 and CAP11 peptides possess LPS- and CD14-binding activities and exert the protective actions in murine endotoxin shock possibly by blocking the binding of LPS to CD14$^+$ cells and suppressing cytokine production by these cells.

Materials and Methods

Reagents

FITC-conjugated LPS (from *Escherichia coli* 0111:B4), LPS (from *E. coli* 0111:B4), 3,3′,5,5′-tetramethyl-benzidine (TMB) liquid substrate system, and D-galactosamine were purchased from Sigma (St. Louis, MO). In some experiments *E. coli* 0111:B4 LPS was biotinylated with biotin-LC-hydrazide, based on the manufacturer's protocol (Pierce, Rockford, IL). Synthetic lipid A LA-15-PP (506) was obtained from Daiichi Pure Chemicals (Tokyo, Japan). A 37-mer peptide of human CAP18 (L^{1}LGDFFRKSKE KIGKEFKRIVQRIKDFLRMLVPRTES^{37}), a guinea pig CAP11 peptide with a free sulfhydryl group of cysteine residue (G^{1}LRKKFRKTRKR IQKLGRKIGKTGRKVWKAWREYGQIPYPCRI^{43}), and a 20-mer peptide of p40-phox, an NADPH oxidase component (L^{1}HITQQDNYSVY NTTPSATQ^{20}) (24) were synthesized by the solid phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by F-moc chemistry. The peptides were eluted from the resin using a standard protocol and purified to homogeneity by reversed phase HPLC on a Cosmosil $5C_{18}$ column (Nacalai Tesqu, Kyoto, Japan), using a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid. The molecular masses of synthesized peptides were confirmed on a mass spectrometer (model TSQ 700; Thermo Quest Finnigan, Manchester, U.K.). To make a dimer form of CAP11, synthesized 43-mer peptide was oxidized in room air. SDS-urea-PAGE analysis revealed that >95% of CAP11 peptide was jointed by disulfide bonding. Human neutrophil peptide-1, human β-defensin (hBD)-1, and hBD-2 were purchased from Peptide Institute (Osaka, Japan). Tissue culture supplies were obtained from Iwaki Glass (Tokyo, Japan).

Antibodies

As anti-LBP Abs, mouse anti-human LBP mAb 6G3 (HyCult Biotechnology, Uden, The Netherlands) and rat anti-mouse LBP mAb clone 39 (class 2) (25) were used. These anti-LBP mAbs can recognize both free LBP and LBP-LPS complexes, and inhibit the binding of LBP-LPS complexes to CD14. As anti-CD14 Abs, FITC-conjugated rat anti-mouse CD14 mAb rmC5-3 (BD PharMingen, San Diego, CA) and FITC-conjugated rat anti-mouse CD14 mAb 4C1 (26) were used. The 4C1 epitope is distinct from the rmC5-3 epitope (aa residues 308–322, the C-terminal moiety of murine CD14). 4C1 can block the binding of LPS to CD14$^+$ cells, whereas rmC5-3 has little effect on LPS binding (26). Ascites of anti-human CD14 mAb MEM-18, which can recognize an epitope in the region spanning aa 57–64 of human CD14 and block the binding of LPS to CD14 (27), was obtained from HyCult Biotechnology. Rabbit control IgG was prepared from non-immunized rabbit serum by affinity chromatography using a Hitrap protein A column (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Cells

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Sanko Junyaku, Tokyo, Japan) at 37°C in 5% CO_2. Confluent RAW264.7 cells were detached by washing with 0.05% EDTA in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4).

Assay for the binding of FITC-conjugated LPS to RAW264.7 cells

RAW264.7 cells (5×10^5 cells/ml) were incubated with FITC-conjugated LPS (100 ng/ml) in the absence or the presence of cathelicidin peptide (CAP18 or CAP11, 0.01–10 μg/ml) in RPMI 1640 containing 10% FBS for 15 min at 37°C. After washing the cells with PBS, the binding of FITC-LPS was analyzed by flow cytometry (FACScan; BD Biosciences, Rutherford, NJ), and median fluorescence intensity was determined. Alternatively, RAW264.7 cells were incubated with FITC-conjugated LPS in the presence of anti-human LBP mAb 6G3 (5 μg/ml), which can cross-react with bovine LBP, in RPMI 1640 containing 10% FBS, and the binding of FITC-LPS was analyzed as described above. In some experiments RAW264.7 cells were preincubated with CAP18 or CAP11 peptide (0.1–10 μg/ml) in RPMI 1640 containing 10% FBS for 10 min at 37°C. After washing, the cells were incubated with FITC-LPS (100 ng/ml) in RPMI 1640 containing 10% FBS for 15 min at 37°C, and LPS binding was evaluated by flow cytometry. Moreover, the binding of FITC-LPS was investigated using RPMI 1640 containing 10% mouse serum. Anti-mouse LBP mAb clone 39 suppressed binding of FITC-LPC to RAW264.7 cells in the medium containing 10% mouse serum, and CAP18 and CAP11 peptides also inhibited LPS binding, as in the medium containing 10% FBS (data not shown).

Evaluation of the expression of TNF-α

RAW264.7 cells (10^6 cells/well in a 24-well microplate) were incubated with LPS (100 ng/ml) in the absence or the presence of cathelicidin peptides (CAP18 or CAP11, 10 μg/ml) in 500 μl of RPMI 1640 containing 10% FBS for 4 h at 37°C. After incubation the cells were detached by washing with 0.05% EDTA/PBS, and the expression of TNF-α mRNA and protein was analyzed by Northern and Western blotting, respectively. In brief, total cellular RNA (2.5 μg) isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method was separated by electrophoresis on 1% agarose-formaldehyde gel and then transferred onto nylon membranes (Hybond N$^+$; Amersham Pharmacia Biotech). RNA was cross-linked with a Funa-UV Linker (Funakoshi, Tokyo, Japan), and the blots were hybridized with cDNA probes, which were labeled with a digoxigenin-high prime DNA labeling kit (Roche, Mannheim, Germany). Probes used were the 0.39-kb TNF-α cDNA (encompassing nucleotides 427–819) (28) obtained by the PCR amplification of LPS-stimulated RAW264.7 cell cDNA, and the 2.1-kb β-actin cDNA (pHFβA-1; provided by P. Gunning and L. Kedes, Stanford University, Palo Alto, CA) (29). In Western blot analysis the cells suspended in PBS containing 1 mM di-isopropyl fluorophosphate were disrupted on ice by sonication, and aliquots (3×10^5 cells for TNF-α and 3×10^4 cells for β-actin) were subjected to SDS-PAGE on a 7.5–20% linear gradient of polyacrylamide under reducing conditions. The resolved proteins were electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA) using a Trans-Blot SD apparatus (Bio-Rad, Hercules, CA). The blots were blocked in Block Ace (Dainippon Pharmaceutical, Osaka, Japan) and probed with rabbit anti-mouse TNF-α Ab (Genzyme, Cambridge, MA) or anti-β-actin mAb (Sigma). The blots were further probed with HRP-conjugated goat anti-rabbit IgG (Organon Teknika, Durham, NC) or goat anti-mouse IgG/IgM (Chemicon, Temecula, CA), and proteins were finally detected with SuperSignal West Pico chemiluminescent substrate (Pierce). In some experiments RAW264.7 cells were incubated with LPS in the medium containing 10% mouse serum, and the effect of cathelicidin peptides on the LPS-induced TNF-α expression was investigated as described above.

Measurement of the LPS-binding activities of cathelicidin peptides

The 96-well microtiter plates (Immulon 2H; Dynex Technologies, Ashford, U.K.) were coated with LPS (100 ng/well) by incubating 50 μl/well of 2 μg/ml LPS in 0.1 M Na_2CO_3 and 20 mM EDTA, pH 9.6, for 3 h at 37°C. The LPS solution was flicked out, and the plates were rinsed thoroughly under running water and air-dried overnight (30). Excess binding sites were blocked with 100 μl/well PBS containing 1% BSA, and CAP18 or CAP11 peptide (0.02–0.1 μg/well) was incubated for 1 h at 37°C in 50 μl of RPMI 1640 without phenol red (Life Technologies, Grand Island, NY). Alternatively, cathelicidin peptide (0.1 μg/well) was incubated in the presence of LPS (0.5–2.5 μg/well) in 50 μl of RPMI 1640. After washing, affinity-purified rabbit anti-CAP18 or anti-CAP11 Ab (50 μl/well; 2 μg/ml in PBS containing 0.1% BSA) was added and incubated for 1 h at 37°C. The Ab solution was then rinsed out, and HRP-conjugated goat anti-rabbit IgG (50 μl/well; diluted 2000-fold in PBS containing 0.1% BSA) was incubated in the wells for 1 h at room temperature. Finally, TMB liquid substrate (100 μl/well) was incubated until sufficient color had developed (5–15 min).

The reaction was stopped by adding 100 μl/well 0.18 M sulfuric acid, and the absorbance at 450 and 560 nm was quantitated in a microtiter plate reader. Anti-CAP18 and anti-CAP11 sera were raised in rabbits with the use of synthetic CAP18 and CAP11 peptides covalently coupled to keyhole limpet hemocyanin, respectively, and Abs were purified by affinity chromatography using the synthetic peptide-conjugated, epoxy-activated, Sepharose (Amersham Pharmacia Biotech).

In addition, the LPS-binding activities of CAP18 and CAP11 peptides were quantified by hemagglutination assay using LPS-sensitized erythrocytes (19). For sensitization, 1% sheep erythrocyte suspension (10 ml; Toyo Bio, Tokyo, Japan) was incubated with 2 ml of 100 μg/ml LPS in saline for 30 min at 37°C. After washing and suspended in PBS, 1% suspension of LPS-sensitized erythrocytes (50 μl) was mixed with a 2-fold serial dilution of cathelicidin peptide (50 μl; 0.31–10 μg/ml in PBS) in the absence or presence of LPS or lipid A (0.06–2.0 μg/ml) in a U-bottom microtiter plate and incubated for 1 h at 37°C. The LPS-binding activity of CAP18 or CAP11 peptide was expressed as a minimum agglutinating concentration (MAC), whereas the inhibition of CAP18- or CAP11-mediated hemagglutination by LPS or lipid A was expressed as a minimum inhibitory concentration (MIC). In some experiments sheep erythrocytes were sensitized with lipid A by incubating with 25 μg/ml lipid A in saline for 30 min at 37°C. After washing, hemagglutination was assayed in saline.

Assay for the interaction of LPS with LBP

LPS (100 ng/well) was immobilized to the 96-well microtiter plates as described above. After blocking, RPMI 1640 containing 0.1, 1, or 10% FBS (50 μl/well) was added and incubated for 1 h at 37°C. The plates were then washed, and 50 μl/well anti-LBP mAb 6G3 (25 nM in PBS containing 0.1% BSA) was incubated in the wells for 1 h at 37°C. The mAb solution was rinsed out and replaced with 50 μl/well HRP-conjugated rabbit anti-mouse IgG (diluted 1000-fold in PBS containing 0.1% BSA; DAKO, Glostrup, Denmark) for 1 h at room temperature. Finally, the binding of LBP to the immobilized LPS was detected by incubating with TMB liquid substrate (100 μl/well). Alternatively, the microtiter plates were preincubated with CAP18 or CAP11 peptide (0.5–2.5 μg/well) in 50 μl/well RPMI 1640 for 1 h at 37°C. After washing, RPMI 1640 containing 10% FBS (50 μl/well) was added, and the binding of LBP was determined as described above. In some experiments the binding of mouse LBP to the immobilized LPS was assessed using mouse serum, rat anti-mouse LBP mAb clone 39, and HRP-conjugated rabbit anti-rat IgG (DAKO).

Assay for the binding of LBP to cathelicidin peptides

The microtiter plates were coated with cathelicidin peptides (2.5 μg/well) by incubating 50 μl/well of 50 μg/ml CAP18 or CAP11 in PBS overnight at room temperature. After blocking, RPMI 1640 containing 10% FBS (50 μl/well) was added and incubated for 1 h at 37°C. The plates were then washed, and 50 μl/well anti-LBP mAb 6G3 (25 nM in PBS containing 0.1% BSA) was incubated in the wells for 1 h at 37°C. The mAb solution was rinsed out and replaced with 50 μl/well HRP-conjugated rabbit anti-mouse IgG (diluted 1000-fold in PBS containing 0.1% BSA) for 1 h at room temperature. Finally, the binding of LBP to the immobilized cathelicidin peptides was detected by TMB reaction. As a positive control, biotinylated LPS (100 ng/well) was added to the CAP18- or CAP11-immobilized plates and incubated for 1 h at 37°C in 50 μl/well RPMI 1640. The LPS solution was then rinsed out, and 50 μl/well HRP-conjugated streptavidin (diluted 5000-fold in PBS containing 0.1% BSA; DAKO) was incubated for 1 h at 37°C. The binding of biotinylated LPS to the immobilized CAP18 or CAP11 was finally detected by TMB reaction.

Flow cytometric assay for the expression of CD14 and the binding of cathelicidin peptides

To analyze the effect of cathelicidin peptides on CD14 expression, RAW264.7 cells (5×10^5 cells/ml) were incubated without or with cathelicidin peptides (CAP18 or CAP11, 10 μg/ml) or LPS (100 ng/ml) in RPMI 1640 containing 10% FBS for 15 min at 37°C and further incubated with FITC-conjugated rat anti-mouse CD14 mAb rmC5-3 (2.5 μg/ml) or FITC-conjugated rabbit anti-mouse IgG (DAKO), as a negative control, for 15 min at 37°C. After washing, the binding of anti-CD mAb was measured by flow cytometry.

To evaluate the binding of cathelicidin peptides to the $CD14^+$ cells, RAW264.7 cells (5×10^5 cells/ml) were incubated with CAP18 or CAP11 (10 μg/ml) in the absence or the presence of CAP11 or CAP18 (20 μg/ml), respectively, in RPMI 1640 containing 10% FBS for 15 min at 37°C. The cells were then washed and incubated with 2 μg/ml rabbit anti-CAP18 or anti-CAP11 Ab or rabbit control IgG, as a negative control, in RPMI 1640 containing 10% FBS for 15 min at 37°C. After washing, the cells were further incubated with FITC-conjugated goat anti-rabbit IgG (diluted 1000-fold in RPMI 1640 containing 10% FBS; Organon Teknika), and the binding of cathelicidin peptides was analyzed by flow cytometry. RAW264.7 cells were also incubated with p40-phox peptide (10 μg/ml), a control peptide, and then the peptide binding was examined using affinity-purified rabbit anti-p40-phox Ab (24) and FITC-conjugated goat anti-rabbit IgG by flow cytometry.

Furthermore, the binding of cathelicidin peptides to CD14 was examined using neutralizing anti-mouse CD14 mAb 4C1 that can recognize the murine CD14 epitope and inhibit the binding of LPS to CD14 (26). RAW264.7 cells (5×10^5 cells/ml) were preincubated without or with cathelicidin peptides (CAP18 or CAP11, 10 μg/ml) or LPS (100 ng/ml), as a positive control, for 15 min at 37°C in RPMI 1640 containing 10% FBS. The cells were then added with 50 ng/ml FITC-conjugated anti-CD14 mAb 4C1 or FITC-conjugated rabbit anti-mouse IgG, as a negative control, and further incubated for 15 min at 37°C. After washing, the binding of FITC-conjugated anti-CD14 mAb 4C1 was analyzed by flow cytometry. Moreover, the binding of FITC-conjugated anti-CD14 mAb 4C1 to RAW264.7 cells was investigated using RPMI 1640 containing 10% mouse serum.

Evaluation of the effect of cathelicidin peptides on murine endotoxin shock model

To determine the protective effect of cathelicidin peptides against the lethal activity of LPS, we used D-galactosamine-sensitized mice that are highly susceptible to LPS (31). Male C57BL/6 mice, aged 10 wk, were purchased from Japan SLC (Shizuoka, Japan). D-galactosamine (18 mg/0.3 ml of saline), FITC-conjugated LPS (100 ng/0.2 ml of saline), and cathelicidin peptides (CAP18 or CAP11, 10 μg/0.2 ml of saline) were sequentially injected i.p. into mice, and deaths were recorded every 24 h until day 6 after the injection. In some experiments, 75 min after LPS challenge mice were sacrificed by drawing blood from the heart, and sera were prepared. Concurrently, peritoneal fluids were harvested by washing the peritoneal cavities with 5 ml of PBS, and the supernatants and pelleted cells (peritoneal macrophages) were recovered. Serum TNF-α levels were determined using a commercially available mouse TNF-α ELISA kit (Endogen, Woburn, MA) that can detect <50 pg/ml TNF-α. Furthermore, using peritoneal macrophages, the binding of FITC-conjugated LPS was analyzed by flow cytometry, and the expression of TNF-α was investigated by Northern blotting and Western blotting, as described in the above sections. In addition, the LBP levels in sera and peritoneal supernatants were quantitated by Western blot analysis using rat anti-mouse LBP mAb clone 39 and HRP-conjugated rabbit anti-rat IgG. To measure the relative amounts, the detected 65-kDa LBP bands were quantified using a scanning densitometer (MasterScan System; Scanalytics, Fairfax, VA), and LBP levels in the peritoneal supernatants were corrected based on the volume of peritoneal fluids recovered.

Statistical analysis

Data are shown as the mean ± SD. Statistical significance was determined by one-way ANOVA with multiple comparison test (StatView; Abacus Concepts, Berkeley, CA), and results were considered significant at $p < 0.05$.

Results

Effects of CAP18 and CAP11 on the binding of FITC-conjugated LPS to RAW264.7 cells

We first examined the effects of cathelicidin peptides (CAP18 and CAP11) on the binding of FITC-conjugated LPS to $CD14^+$ cells by flow cytometry using the murine macrophage cell line RAW264.7. When FITC-LPS was incubated with RAW264.7 cells, it bound to the cells serum-dependently (Fig. 1, *upper panel*); FITC-LPS was hardly bound to the cells in the absence of serum, and the fluorescence intensity was almost the same as that of background without FITC-LPS. Furthermore, FITC-LPS was incubated with RAW264.7 cell in the presence of anti-LBP mAb (5 μg/ml) that can recognize both free LBP and LBP-LPS complexes and inhibit the binding of LBP-LPS complexes to CD14. The anti-LBP mAb inhibited the binding of FITC-LPS to RAW264.7 cells by 96.0 ± 3.5% ($n = 3$), indicating that the LPS binding requires serum LBP.

Interestingly, CAP18 and CAP11 (10 μg/ml each) markedly suppressed the binding of FITC-LPS to RAW264.7 cells (Fig. 1, *lower panel*). Their effects were dose dependent (Fig. 2), and

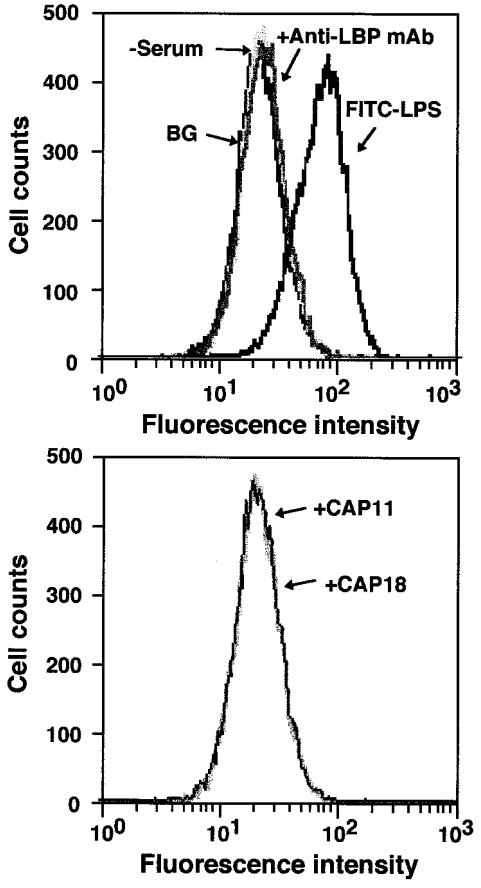

FIGURE 1. Effect of CAP18 and CAP11 on the binding of FITC-conjugated LPS to RAW264.7 cell. RAW264.7 cells (5×10^5 cells/ml) were incubated with 100 ng/ml FITC-conjugated LPS (FITC-LPS) in the absence or the presence of 10 μg/ml CAP18 (+CAP18) or CAP11 (+CAP11) peptide in RPMI 1640 containing 10% FBS for 15 min at 37°C. Alternatively, RAW264.7 cells were incubated with FITC-conjugated LPS in the presence of 5 μg/ml anti-LBP mAb 6G3 (+Anti-LBP mAb) in RPMI 1640 containing 10% FBS or with FITC-LPS in RPMI 1640 without 10% FBS (−Serum). After washing, the binding of FITC-LPS was analyzed by flow cytometry. Background was assessed by using RAW264.7 cells incubated without FITC-LPS. Data are from one of three separate experiments.

CAP11 (IC_{50} = 0.05 μg/ml or 0.005 μM) was more potent than CAP18 (IC_{50} = 0.25 μg/ml or 0.056 μM) in inhibiting the binding of FITC-LPS to RAW264.7 cells. Furthermore, the effect of CAP18 and CAP11 on the TNF-α expression was examined by Northern and Western blot analyses (Fig. 3). CAP18 and CAP11 (10 μg/ml each) completely suppressed LPS-induced TNF-α expression by RAW264.7 cells at both mRNA and protein levels.

LPS-binding activities of CAP18 and CAP11 and their effects on the binding of LPS to LBP

To clarify the mechanisms by which CAP18 and CAP11 inhibit the binding of LPS to CD14$^+$ cells, we investigated the LPS-binding activities of CAP18 and CAP11 using LPS-immobilized microtiter plates. CAP18 and CAP11 bound to the LPS-immobilized plates in a dose-dependent fashion (Fig. 4*A*), and the binding was dose-dependently inhibited by LPS added to the plates (Fig. 4*B*). Noticeably, the binding of CAP11 was more potently inhibited by LPS (IC_{50} = 0.07 μg LPS/well) compared with that of CAP18 (IC_{50} = 0.28 μg LPS/well).

In addition, the LPS-binding activities were quantified by hemagglutination assay using LPS-sensitized sheep erythrocytes.

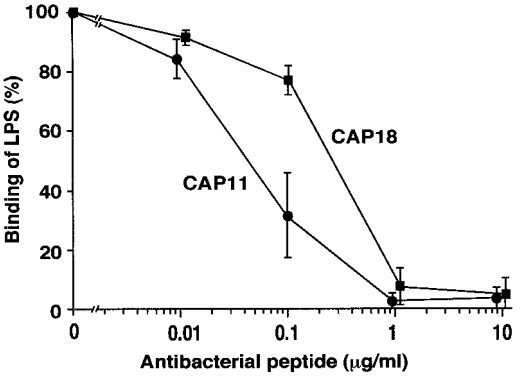

FIGURE 2. Dose-dependent inhibition of binding of FITC-conjugated LPS to RAW264.7 cells by CAP18 and CAP11. RAW264.7 cells (5×10^5 cells/ml) were incubated with 100 ng/ml FITC-conjugated LPS in the absence or presence of 0.01–10 μg/ml CAP18 or CAP11 in RPMI 1640 containing 10% FBS for 15 min at 37°C. After washing, the binding of FITC-LPS was analyzed by flow cytometry, and median fluorescence intensity was determined. Binding of LPS was expressed as a percentage of that obtained using RAW264.7 cells incubated with FITC-LPS in the absence of CAP18 or CAP11. Data are the mean ± SD of three to five separate experiments.

CAP18 and CAP11 agglutinated the LPS-sensitized erythrocytes, with MACs of 2.5 and 0.625 μg/ml (0.568 and 0.059 μM), respectively. Furthermore, the addition of LPS inhibited the CAP18- and CAP11-induced agglutination of LPS-sensitized erythrocytes, with MICs of 1.0 and 0.25 μg/ml, respectively. These observations indicate that both CAP18 and CAP11 have LPS-binding activities, and CAP11 has more potent LPS-binding activity than CAP18. Moreover, the addition of lipid A inhibited the CAP18- and CAP11-induced agglutination of LPS-sensitized erythrocytes, with MICs of 0.5 and 0.125 μg/ml, respectively, suggesting that CAP18 and CAP11 can recognize the lipid A moiety of LPS. Actually, CAP18 and CAP11 could agglutinate the lipid A-sensitized erythrocytes, with MACs of 2.5 and 0.625 μg/ml, respectively.

Using the LPS-immobilized plates, we next examined the effects of CAP18 and CAP11 on the binding of LPS to LBP that catalyzes the transfer of LPS to CD14. When the LPS plates were incubated

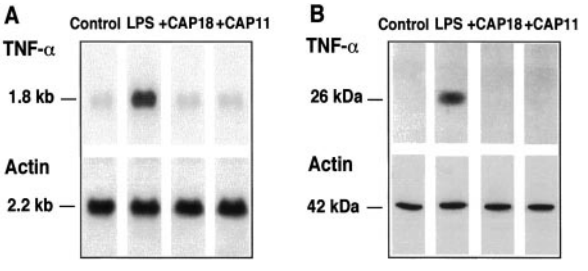

FIGURE 3. Effect of CAP18 and CAP11 on LPS-induced TNF-α expression by RAW264.7 cells. RAW264.7 cells (10^6 cells/well in a 24-well microplate) were incubated without (Control) or with 100 ng/ml LPS in the absence or the presence of 10 μg/ml CAP18 (+CAP18) or CAP11 (+CAP11) in 500 μl of RPMI 1640 containing 10% FBS for 4 h at 37°C. After incubation, the cells were recovered and the expression of TNF-α mRNA and protein was analyzed by Northern and Western blotting, respectively. *A*, Total cellular RNA was separated by electrophoresis on 1% agarose-formaldehyde gel, and Northern blotting was performed using digoxigenin-labeled mouse TNF-α cDNA or β-actin cDNA probe. *B*, Cell aliquots were subjected to SDS-PAGE on a 7.5–20% linear gradient of polyacrylamide under reducing conditions, and Western blotting was performed using rabbit anti-mouse TNF-α Ab or anti-β-actin mAb. Data are from one of three separate experiments.

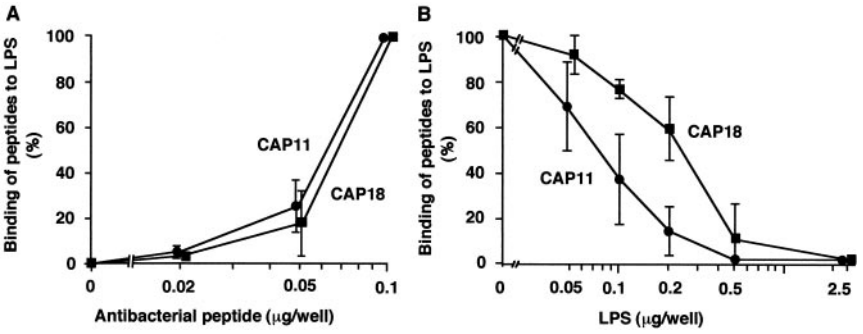

FIGURE 4. Evaluation of the LPS-binding activities of CAP18 and CAP11. *A*, The LPS-binding activities of cathelicidin peptides were investigated by incubating 0.02–0.1 μg of CAP18 or CAP11 in the LPS-immobilized 96-well microtiter plates (100 ng LPS/well) for 1 h at 37°C in 50 μl of RPMI 1640. The bound peptides were detected by TMB reaction using rabbit anti-CAP18 or anti-CAP11 Ab and HRP-conjugated goat anti-rabbit IgG. *B*, CAP18 or CAP11 (0.1 μg each) was incubated in the LPS-immobilized 96-well microtiter plates for 1 h at 37°C in the absence or the presence of LPS (0.5–2.5 μg/well) in 50 μl of RPMI 1640, and the bound peptides were detected as described above. Binding of peptides to the LPS-immobilized plates was expressed as a percentage of that incubated with 0.1 μg of each peptide in the absence of added LPS. Data are the mean ± SD of three separate experiments.

with FBS, serum LBP bound to the LPS plates dose-dependently on the concentrations of serum used (Fig. 5*A*). Moreover, the LPS plates were pretreated with CAP18 or CAP11, and then the LPS-LBP interaction was analyzed. The binding of LBP to the LPS plates was inhibited in a dose-dependent manner by CAP18 and CAP11 added to the plates (Fig. 5*B*). Importantly, CAP11 (IC_{50} = 0.06 μg/well or 5.7 pmol/well) was more potent than CAP18 (IC_{50} = 0.2 μg/well or 44.5 pmol/well) in inhibiting LPS-LBP binding.

Furthermore, we evaluated the interaction of LBP with cathelicidin peptides using CAP18- or CAP11-immobilized microtiter plates. Apparently, substantial amounts of biotinylated LPS, a positive control, could bind to the CAP18 or CAP11 plates. On the contrary, the binding of LBP to the plates was hardly detected (data not shown).

These above observations indicate that both CAP18 and CAP11 can bind to LPS, but not LBP, thereby inhibiting the interaction of LPS with LBP. Moreover, CAP11 has more potent activities than CAP18 for binding to LPS and inhibiting LPS/LBP interaction.

Effect of CAP18 and CAP11 on CD14 expression, and binding of CAP18 and CAP11 to CD14$^+$ cells

It is possible that cathelicidin peptides may alter CD14 expression, thereby affecting the binding of LPS to $CD14^+$ cells. To check this, we investigated the expression of CD14 after treatment of RAW264.7 cells with CAP18 or CAP11 by flow cytometry using FITC-conjugated anti-mouse CD14 mAb rmC5-3. Neither CAP18, CAP11 (10 μg/ml each), nor LPS (100 ng/ml) changed the CD14 expression on RAW264.7 cells (Fig. 6).

Likewise, it is feasible that cathelicidin peptides bind to the CD14 molecule on the cells and influence the binding of LPS to $CD14^+$ cells without affecting CD14 expression. To confirm this, we preincubated RAW264.7 cells with CAP18 or CAP11, and after washing we analyzed the binding of FITC-LPS by flow cytometry. Interestingly, the preincubation with CAP18 or CAP11 inhibited the binding of FITC-LPS to RAW264.7 cells (Fig. 7). The inhibition was dose dependent on the peptides used, and CAP11 (IC_{50} = 1.4 μg/ml or 0.133 μM) was more potent than CAP18 (IC_{50} = 6 μg/ml or 1.362 μM) in inhibiting the binding of FITC-LPS to RAW264.7 cells. These observations indicate that CAP18 and CAP11 can bind to the cells and affect LPS binding. In fact, flow cytometric assay using anti-CAP18 and anti-CAP11 Abs revealed that CAP18 and CAP11 could bind to RAW264.7 cells (Fig. 8*A*). On the contrary, p40-phox peptide (10 μg/ml), a control peptide, did not essentially bind to the cells (data not shown). Of interest, CAP11 (20 μg/ml) inhibited the binding of CAP18 (10 μg/ml) to RAW264.7 cells by 72.1 ± 5.1% (n = 3). Conversely, CAP18 (20 μg/ml) inhibited the binding of CAP11 (10 μg/ml) to the cells by 72.2 ± 14.1% (n = 3). These observations indicate that CAP18 and CAP11 specifically bind to RAW264.7 cells, and the two peptides may share the same receptors (binding sites) on the cells.

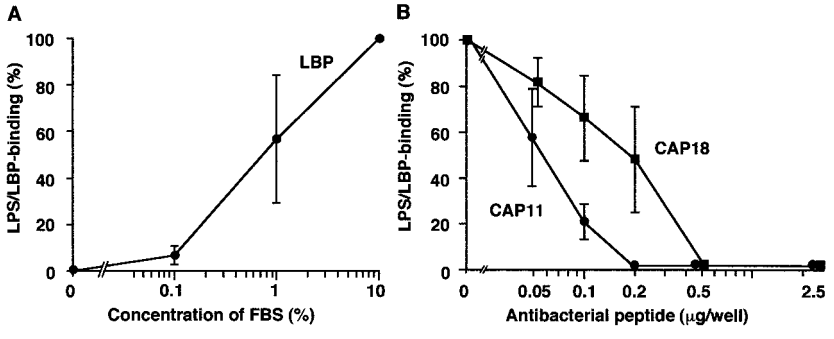

FIGURE 5. Effect of CAP18 and CAP11 on the interaction of LPS with LBP. *A*, The LPS/LBP binding was examined by incubating 50 μl of RPMI 1640 containing 0.1, 1, or 10% FBS in the LPS-immobilized 96-well microtiter plates (100 ng LPS/well) for 1 h at 37°C. After incubation, the bound LBP was detected by TMB reaction using anti-LBP mAb 6G3 and HRP-conjugated rabbit anti-mouse IgG. *B*, The LPS-immobilized microtiter plates were preincubated with 0.5–2.5 μg/well CAP18 or CAP11 in 50 μl of RPMI 1640 for 1 h at 37°C. Thereafter, LPS/LBP binding was determined by incubating 50 μl of RPMI 1640 containing 10% FBS in the microtiter plates as described above. LPS/LBP binding was expressed as a percentage of that incubated with RPMI 1640 containing 10% FBS in the absence of added CAP18 or CAP11. Data are the mean ± SD of three to six separate experiments.

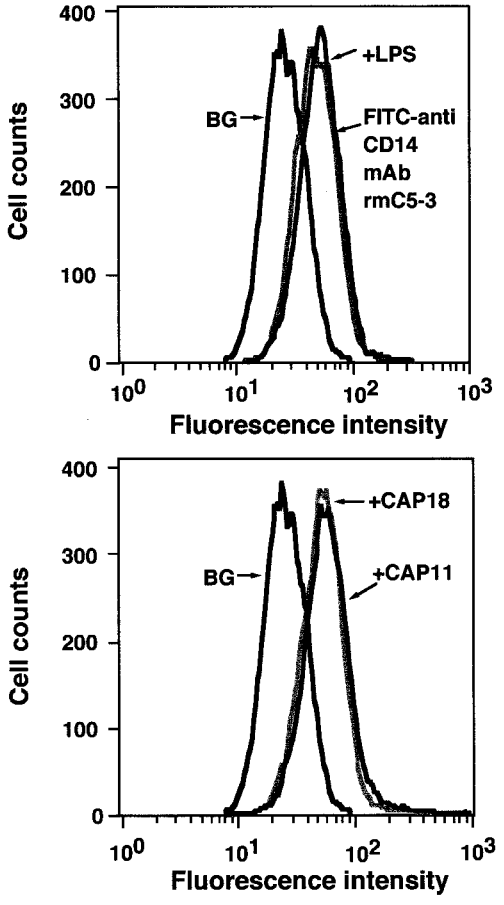

FIGURE 6. Effect of CAP18 and CAP11 on the expression of CD14 by RAW264.7 cells. RAW264.7 cells (5×10^5 cells/ml) were incubated without or with 10 μg/ml CAP18 (+CAP18) or CAP11 (+CAP11), or 100 ng/ml LPS (+LPS) in RPMI 1640 containing 10% FBS for 15 min at 37°C, and further incubated with 2.5 μg/ml FITC-conjugated rat anti-mouse CD14 mAb rmC5-3 (FITC-anti-CD14 mAb rmC5-3) for 15 min at 37°C. After washing, the binding of anti-CD mAb was measured by flow cytometry. Background was assessed by using RAW264.7 cells incubated with FITC-conjugated rabbit anti-mouse IgG (a negative control for nonspecific binding). Data are from one of three separate experiments.

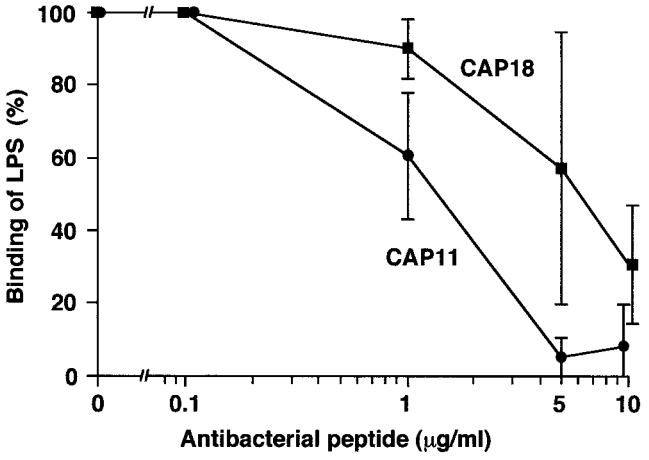

FIGURE 7. Inhibition of the binding of FITC-conjugated LPS to RAW264.7 cells by preincubation with CAP18 and CAP11. RAW264.7 cells (5×10^5 cells/ml) were preincubated with 0.1–10 μg/ml CAP18 or CAP11 in RPMI 1640 containing 10% FBS for 10 min at 37°C. After washing, the cells were incubated with 100 ng/ml FITC-conjugated LPS in RPMI 1640 containing 10% FBS for 15 min at 37°C, and the binding of FITC-LPS was analyzed by flow cytometry. LPS binding was expressed as a percentage of that obtained using RAW264.7 cells preincubated without CAP18 or CAP11. Data are the mean ± SD of three to six separate experiments.

To further determine the binding of cathelicidin peptides to CD14, we used anti-CD14 mAb 4C1 that can recognize the murine CD14 epitope and inhibit the binding of LPS to CD14$^+$ cells (26). First, RAW264.7 cells were preincubated with LPS (100 ng/ml), and the binding of FITC-conjugated anti-CD14 mAb 4C1 (50 ng/ml) to the cells was analyzed. As expected, preincubation with LPS suppressed the binding of anti-CD14 mAb 4C1 to RAW264.7 cells by 47.9 ± 5.2% ($n = 5$; Fig. 8*B*), suggesting that the epitope, which can be recognized by mAb 4C1, is located on murine CD14 next to the LPS binding site. Next, RAW264.7 cells were preincubated with CAP18 or CAP11 (10 μg/ml each), and the binding of FITC-conjugated anti-CD14 mAb 4C1 to the cells was determined. Similar to the results with LPS, preincubation with CAP18 or CAP11 inhibited the binding of FITC-conjugated anti-CD14 mAb 4C1 to RAW264.7 cells by 43.0 ± 10.6% ($n = 4$) and 56.8 ± 10.3% ($n = 3$), respectively (Fig. 8*C*). These observations suggest that CAP18 and CAP11 can bind to CD14 near the LPS binding site and influence the binding of LPS to CD14$^+$ cells.

In separate experiments we confirmed using 10% mouse serum-containing medium that CAP18 and CAP11 could suppress the binding of FITC-conjugated LPS to RAW264.7 cells, LPS-induced TNF-α expression by RAW264.7 cells, binding of LBP to LPS, and binding of FITC-conjugated anti-CD14 mAb 4C1 to RAW264.7 cells, as observed with 10% FBS-containing medium (data not shown). These observations indicate that CAP18 and CAP11 could function in mice.

Effects of CAP18 and CAP11 on the murine endotoxin shock model

Using D-galactosamine-sensitized mice, we assessed the actions of cathelicidin peptides on the lethal activity of LPS in vivo (31). D-galactosamine administration sensitized mice to the lethal effect of LPS, and 90% of the sensitized mice died within 24 h after the i.p. injection of 100 ng of LPS (Fig. 9*A*). Noticeably, the administration of CAP18 or CAP11 (10 μg/mouse) increased the survival rate to 80 and 100%, respectively. Moreover, CAP18 and CAP11 administration markedly lowered the LPS-induced increase in serum TNF-α levels ($p < 0.05$; Fig. 9*B*). In addition, the effect of cathelicidin peptides on the binding of FITC-conjugated LPS to peritoneal macrophages (CD14$^+$ cells) was analyzed by flow cytometry. CAP18 or CAP11 administration significantly suppressed the binding of FITC-LPS to peritoneal macrophages ($p < 0.001$; Fig. 10). Concurrently, TNF-α expression was investigated using peritoneal macrophages. Northern and Western blot analyses indicated that CAP18 or CAP11 administration markedly suppressed LPS-induced TNF-α mRNA and protein expression by peritoneal macrophages (Fig. 11).

In separate experiments we confirmed in vitro that FITC-conjugated LPS was able to bind to peritoneal macrophages in the medium containing >1% mouse serum, and LPS binding was suppressed not only by anti-mouse LBP mAb clone 39, but also by CAP18 and CAP11 (data not shown). Furthermore, we found by Western blotting that the LBP levels in the peritoneal supernatants were apparently the same among different groups of endotoxin shock model mice (D-galactosamine administration alone or with LPS with or without CAP18 or CAP11), and were 10.3 ± 2.7% ($n = 16$) of those in the sera. Together these observations suggest that CAP18 and CAP11 could block the LBP-mediated transport

of LPS to CD14$^+$ cells (peritoneal macrophages) in vivo, thereby suppressing the production of TNF-α by these cells.

Discussion

LPS, also known as endotoxin, is the outer membrane component of Gram-negative bacteria, which has an extremely biologically activity and a key role in the pathogenesis of endotoxin shock associated with the septic syndrome (1, 32). LPS activates mononuclear phagocytes (monocytes and macrophages) and other types of cells to produce and release potent inflammatory mediators, of which TNF-α appears to be very important for the development of endotoxin shock (2–4). Endotoxin shock could occur in the process of antibiotic therapy for the underlying bacterial infections in septic syndrome. In this context, some antibiotics are known to kill the bacteria but stimulate the release of LPS from the outer membrane of the dying bacteria, thereby provoking the occurrence of endotoxin shock (33, 34); they cannot neutralize the biological activity of released LPS. Thus, a drug that suppresses the actions of LPS could be a reasonable additional therapy for endotoxin shock or sepsis caused by Gram-negative bacterial infections. Until now, several strategies, including neutralizing Abs against LPS, LBP, or cytokines, have been tested to prevent the cascade of LPS-induced inflammatory reactions (2, 25, 35–37). Recently, however, much attention has focused on the low m.w. cationic antibacterial peptides that possess both the antibacterial and LPS-neutralizing activities. Originally, those peptides are known to function in the innate host defense against microbial infections by impairing the membranes of targeted organisms (12–15).

Scott et al. (38, 39) synthesized the hybrid peptides derived from silk moth cecropin and bee melittin peptides and examined their effects on the binding of LPS to LBP. Furthermore, Iwagaki et al. (40) investigated the binding of LPS to CD14$^+$ cells using synthetic antiendotoxin peptides that were designed to mimic the structures of polymyxin B, which inhibits the biological activities of LPS through its high affinity binding to the lipid A moiety of LPS. In addition, Dankesreiter et al. (41) synthesized the hybrid peptides containing LPS-binding domains from *Limulus* anti-LPS factor and LBP and examined their effects on the interaction of LPS with CD14$^+$ cells. However, the detailed mechanisms by which those synthetic peptides exert the LPS-neutralizing activities are not fully understood, and it is unclear whether those synthetic peptides could influence the CD14 (LPS receptor) expression and/or bind to CD14 to affect the LPS binding. Furthermore, the information is very limited about the LPS-neutralizing activities of naturally occurring antibacterial peptides in mammals.

Recently, it has been reported that a peptide derived from human CAP18, a member of cathelicidins, can lower serum TNF-α levels and protect mice from lethal endotoxin shock (22). In this study to clarify the possible mechanisms for the protective actions of cathelicidin peptides, we evaluated the LPS-neutralizing activities of human CAP18 and guinea pig CAP11 peptides using the CD14$^+$ murine macrophage cell line RAW264.7 and the murine endotoxin shock model. Flow cytometric analysis revealed that CAP18 and CAP11 inhibited the binding of FITC-conjugated LPS to RAW264.7 cells. Likewise, Northern and Western blot analyses indicated that CAP18 and CAP11 suppressed LPS-induced TNF-α mRNA and protein expression by RAW264.7 cells. Interestingly, CAP18 and CAP11 possessed LPS- or lipid A-binding activities, and they strongly suppressed the interaction of LPS with LBP that transports LPS to CD14. Moreover, CAP18 and CAP11 could bind to CD14 and inhibited the binding of FITC-LPS to the CD14$^+$ cells, although they did not alter CD14 expression on the cells. Furthermore, CAP18 and CAP11 administration inhibited LPS binding to CD14$^+$ cells (peritoneal macrophages) and suppressed

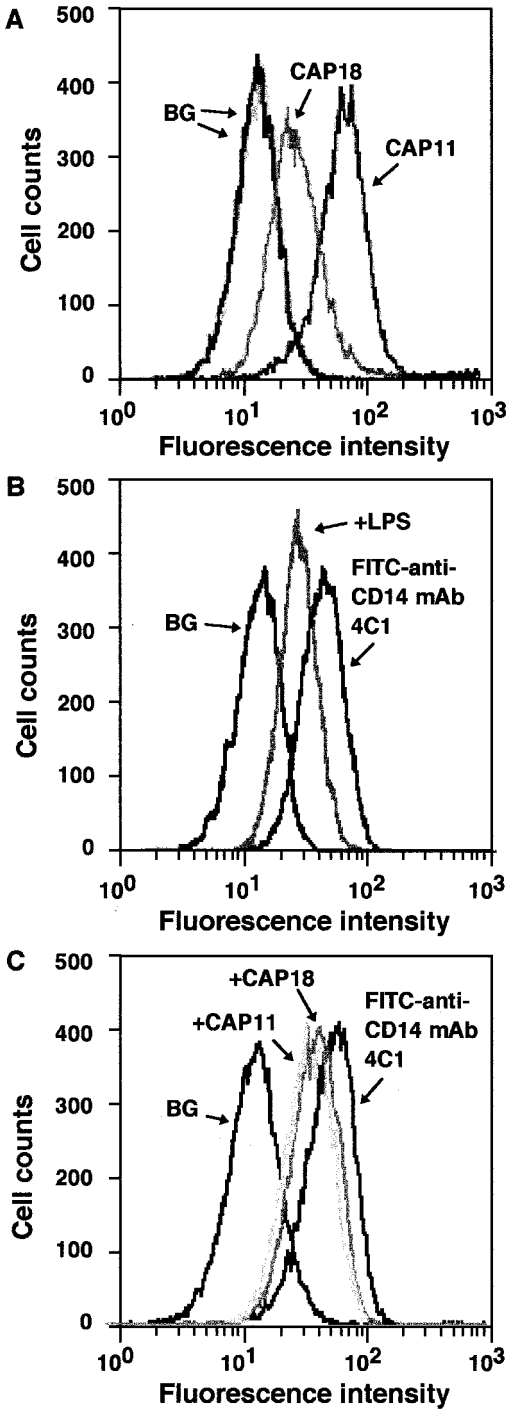

FIGURE 8. Evaluation of the binding of CAP18 and CAP11 to CD14$^+$ cells. *A*, RAW264.7 cells (5×10^5 cells/ml) were incubated with 10 μg/ml CAP18 or CAP11 in RPMI 1640 containing 10% FBS for 15 min at 37°C. After washing, the binding of the peptides was detected using rabbit anti-CAP18 or anti-CAP11 Ab and FITC-conjugated goat anti-rabbit IgG. Background was assessed by using RAW264.7 cells that were treated with CAP18 or CAP11 and then incubated with rabbit control IgG and FITC-conjugated goat anti-rabbit IgG. *B* and *C*, RAW264.7 cells (5×10^5 cells/ml) were preincubated without or with 100 ng/ml LPS (+LPS) or with 10 μg/ml CAP18 or CAP11 (+CAP18 or +CAP11) for 15 min at 37°C in RPMI 1640 containing 10% FBS. The cells were further incubated with 50 ng/ml FITC-conjugated neutralizing anti-CD14 mAb 4C1 (FITC-anti-CD14 mAb 4C1) for 15 min at 37°C, and after washing, the binding of anti-CD14 mAb 4C1 was analyzed by flow cytometry. Background was assessed by using RAW264.7 cells incubated with FITC-conjugated rabbit anti-mouse IgG (a negative control for nonspecific binding). Data are from one of three to five separate experiments.

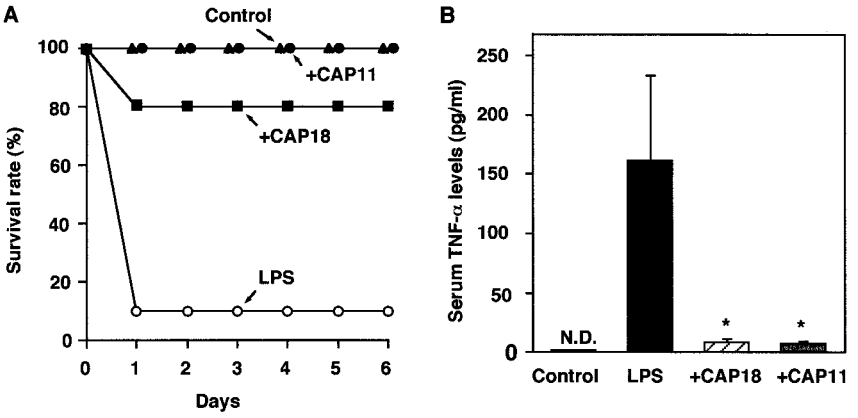

FIGURE 9. Protective actions of CAP18 and CAP11 on the survival of and serum TNF-α levels in D-galactosamine-sensitized mice challenged with LPS. *A*, Mice were i.p. injected with 18 mg/mouse D-galactosamine alone (Control) or with 100 ng/mouse FITC-conjugated LPS with or without 10 μg/mouse of CAP18 (+CAP18) or CAP11 (+CAP11). After the injection, deaths were recorded every 24 h until day 6 (eight mice in each group). *B*, After LPS challenge (75 min), sera were prepared, and serum TNF-α levels were determined using a commercially available mouse TNF-α ELISA kit. Data are the mean $\pm$ SD of eight mice in each group. N.D., Not detected. TNF-α levels are compared without and with CAP18 or CAP11 administration. $*$, $p < 0.05$.

LPS-induced TNF-α expression by these cells in murine endotoxin shock model. Among TLRs, TLR4 is reported to be required for the LPS-CD14 complex to initiate intracellular signaling and induce inflammatory responses (42, 43). Together these observations indicate that cathelicidin peptides CAP18 and CAP11 could exert the protective actions in murine endotoxin shock by blocking the LBP-mediated transport of LPS to CD14$^+$ cells, thereby possibly suppressing the TLR4-mediated cytokine (such as TNF-α) production by these cells via their potent binding activities for LPS and CD14.

Previously, the peptides derived from human and rabbit CAP18, members of the cathelicidin family, were shown to have LPS-binding activities (18, 19). In this study we have revealed that a 37-mer peptide of human CAP18 and CAP11 (a guinea pig homologue of CAP18) possesses LPS- or lipid A-binding activities and can suppress the biological actions of LPS. Thus, among cathelicidin peptides, only CP18-derived peptides and CAP11 are shown to have LPS-binding and neutralizing activities. LPS and lipid A, which entirely contains the endotoxic activities of LPS, are amphipathic molecules with negative charges, and their negatively charged groups (phosphate groups and carboxyl groups of 2-keto-3-deoxyoctulosonic acid, Kdo) are important for the expression of biological activities (32). Interestingly, the potential LPS binding domain of LPS-neutralizing proteins such as *Limulus* anti-LPS factor, LBP, and bactericidal/permeability-increasing protein contains a positively charged amphipathic structure, and the positively charged groups of the LPS-binding domain are thought to interact with the negatively charged groups of lipid A and LPS (44). Consistent with this, CAP18 and CAP11 peptides display the amphipathic α helical structure with hydrophilic (positively charged) and hydrophobic surfaces (20). Thus, the structural feature of CAP18 and CAP11 peptides (positively charged amphipathic structure) is considered important for their LPS-binding capacities. Importantly, CAP11 exhibited more potent LPS-neutralizing activities than CAP18, and pI values for CAP18 and CAP11 were 11.12 and 12.31, respectively (Genetyx-Mac computer system; Software Development, Tokyo, Japan). These observations indicate that the

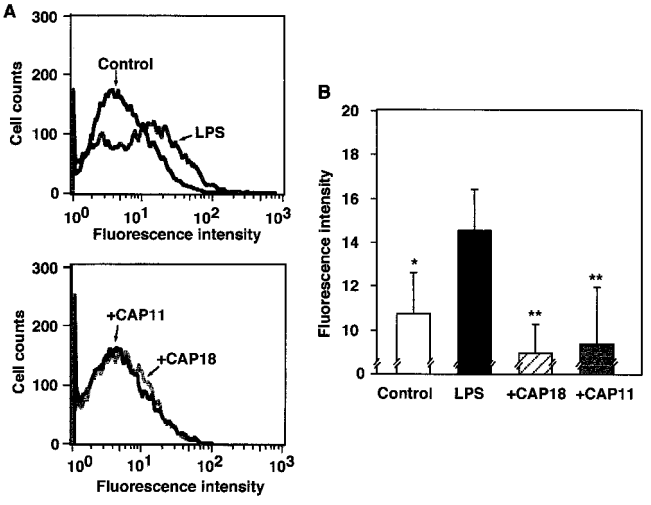

FIGURE 10. Effect of CAP18 and CAP11 administration on the binding of FITC-conjugated LPS to mouse peritoneal macrophages. Mice were i.p. injected with D-galactosamine alone (Control) or with FITC-conjugated LPS with or without CAP18 (+CAP18) or CAP11 (+CAP11) as described in Fig. 9. After LPS challenge (75 min), peritoneal macrophages were recovered, and the binding of FITC-conjugated LPS to the cells was analyzed by flow cytometry. *A*, Data are from one of eight mice in each group. *B*, Data are the mean $\pm$ SD of eight mice in each group. Values are compared without and with CAP18 or CAP11 administration and also with and without LPS administration. $*$, $p < 0.05$; $**$, $p < 0.001$.

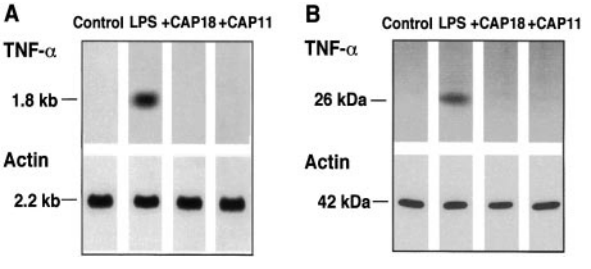

FIGURE 11. Effect of CAP18 and CAP11 administration on LPS-induced TNF-α expression by mouse peritoneal macrophages. Mice were i.p. injected with D-galactosamine alone (Control) or with FITC-conjugated LPS with or without CAP18 (+CAP18) or CAP11 (+CAP11) as described in Fig. 9. After LPS challenge (75 min), peritoneal macrophages were recovered, and the expression of TNF-α mRNA and protein was analyzed by Northern and Western blotting, respectively, as described in Fig. 3. *A*, Western blotting was performed using rabbit anti-mouse TNF-α Ab or anti-β-actin mAb. *B*, Northern blotting was performed using digoxigenin-labeled mouse TNF-α cDNA or β-actin cDNA probe. Data are from one of eight mice in each group.

cationicities of antibacterial peptides may correlate with their LPS-binding activities. Supporting this, we have demonstrated that human α-defensin (human neutrophil peptide-1) and β-defensins (hBD-1 and hBD-2) with pI values of 8.28, 8.55, and 9.25, respectively, have much lower LPS-binding activities than CAP18 and CAP11, as assessed by LPS-sensitized hemagglutination assay, and they can have little inhibitory effect on the binding of FITC-conjugated LPS to RAW264.7 cells or the suppression of LPS-induced TNF-α expression by these cells (data not shown). However, in contrast to the amphipathic α helical structure of CAP18 and CAP11 peptides, defensins exhibit the positively charged amphipathic β-sheet structure (12, 14, 15), suggesting that these structures may be also involved in the difference between LPS-neutralizing activities of defensins and cathelicidin peptides (CAP18 and CAP11).

Using a neutralizing anti-mouse CD14 mAb 4C1, we have indicated that CAP18 and CAP11 can bind to murine CD14 and inhibit the binding of LPS to $CD14^+$ cells. However, neither an epitope for 4C1 nor an LPS binding site has been identified in murine CD14 (26). In contrast, it has been demonstrated that LPS can bind to the negatively charged domain in human CD14 spanning aa 57–64 (D^{57}ADPRQYA^{64}), which can be recognized by a neutralizing anti-human CD14 mAb MEM-18 (27). Moreover, it has been reported that the region at position 9–13 (D^9DEDF^{13}) comprising anionic amino acids is also involved in the binding of LPS to human CD14 (45). In separate experiments we investigated the effect of CAP18 and CAP11 on the binding of anti-CD14 mAb MEM-18 to CD14 using CD14-expressing U937 cells that had been differentiated by incubation with 10 nM PMA for 2 days (46). Importantly, CAP18 and CAP11 (10 μg/ml) inhibited the binding of MEM-18 (1/20 dilution of ascites) to $CD14^+$ U937 cells by 50–80% (data not shown), indicating that CAP18 and CAP11 are able to bind to the LPS binding site on human CD14. Thus, it is tempting to speculate that CAP18 and CAP11 can bind to the negatively charged LPS-binding domain(s), if any, present in murine CD14, thereby inhibiting the binding of LPS to mouse $CD14^+$ cells. Supporting this, the amino acid sequence homology search (Genetyx-Mac computer system) has revealed that murine CD14 contains two negatively charged regions, D^{11}EESC^{15} and E^{57}ADLGQFT^{64}, corresponding to the D^9DEDF^{13} and D^{57}ADPRQYA^{64} sequences of human CD14, respectively (47). However, it has not been determined whether the two regions of murine CD14 are involved in LPS binding. Moreover, CAP18 and CAP11 peptides could only partially (at most ~50%) inhibit the binding of anti-CD14 mAb 4C1 to $CD14^+$ RAW264.7 cells, and the exact binding site(s) for CAP18 and CAP11 in murine CD14 has not been defined. Thus, the mechanism by which the cell surface-bound peptides suppress the binding of LPS to CD14 molecule remains speculative and needs further experimental development in the future.

Human CAP18 and guinea pig CAP11 peptides can exhibit the antibacterial activities against Gram-negative and Gram-positive bacteria in the extracellular milieu containing a physiological concentration of NaCl (150 mM), whereas the antibacterial activities of defensins are completely lost under these conditions (48). Moreover, we have argued in this study that CAP18 and CAP11 could bind to LPS and CD14 and neutralize the activities of LPS. To suppress the cascade of LPS-induced inflammatory reactions, several agents, including neutralizing Abs against LPS, LBP, or cytokines, have been examined (2, 25, 35–37). Unlike these agents, CAP18 and CAP11 would be expected not only to block the onset of LPS-triggered inflammatory reactions by binding directly to LPS and CD14, but also to overcome the underlying Gram-negative bacterial infections in the septic shock syndrome. Thus, CAP18, CAP11, and their derivatives could be attractive candidates for adjunctive therapy in Gram-negative bacterial sepsis.

Acknowledgments

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References

1. Morrison, D. C., R. L. Danner, C. A. Dinarello, R. S. Munford, C. Natanson, M. Pollack, J. J. Spitzer, R. J. Ulevith, S. N. Vogel, and E. McSweegan. 1994. Bacterial endotoxins and pathogenesis of Gram-negative infections: current status and future direction. *J. Endotoxin Res. 1:71.*
2. Beutler, B., I. W. Milsark, and A. C. Cerami. 1985. Passive immunization against cathectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:869.*
3. Lehmann, V., M. A. Freudenberg, and C. Galanos. 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J. Exp. Med. 165:657.*
4. Cerami, A., and B. Beutler. 1988. The role of cathectin/TNF in endotoxic shock and cachexia. *Immunol. Today 9:28.*
5. Kopp, E., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol. 11:13.*
6. Ulevitch, R. J., and P. S. Tobias. 1999. Recognition of Gram-negative bacteria and endotoxin by the innate immune system. *Curr. Opin. Immunol. 11:19.*
7. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature 406:782.*
8. Brightbill, H. D., and R. L. Modlin. 2000. Toll-like receptors: molecular mechanisms of the mammalian immune response. *Immunology 101:1.*
9. Fenton, M. J., and D. T. Golenbock. 1998. LPS-binding proteins and receptors. *J. Leukocyte Biol. 64:25.*
10. Schumann, R. R., and E. Latz. 2000. Lipopolysaccharide-binding protein. *Chem. Immunol. 74:42.*
11. Kitchens. R. L. 2000. Role of CD14 in cellular recognition of bacterial lipopolysaccharides. *Chem. Immunol. 74:61.*
12. Ganz, T., and R. I. Lehrer. 1998. Antimicrobial peptides of vertebrates. *Curr. Opin. Immunol. 10:41.*
13. Gudmundsson, G. H., and B. Agerberth. 1999. Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. *J. Immunol. Methods 232:45.*
14. Hancock, R. E. W., and G. Diamond. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol. 8:402.*
15. Levy, O. 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood 96:2664.*
16. Tsutsumi-Ishii, Y., T. Hasebe, and I. Nagaoka. 2000. Role of CCAAT/enhancer-binding protein site in transcription of human neutrophil peptide-1 and -3 defensin genes. *J. Immunol. 164:3264.*
17. Zanetti, M., Gennaro, R., and Romeo, D. 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett. 374:1.*
18. Hirata, M., Y. Shimomura, M. Yoshida, J. G. Morgan, I. Palings, D. Wilson, M. H. Yen, S. C. Wright, and J. W. Larrick. 1994. Characterization of a rabbit cationic protein (CAP18) with lipopolysaccharide-inhibitory activity. *Infect. Immun. 62:1421.*
19. Larrick, J. W., M. Hirata, R. F. Balint, J. G. Lee, J. Zhong, and S. C. Wright. 1995. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect. Immun. 63:1291.*
20. Yomogida, S., I. Nagaoka, and T. Yamashita. 1996. Purification of the 11- and 5-kDa antibacterial polypeptides from guinea pig neutrophils. *Arch. Biochem. Biophys. 328:219.*
21. Nagaoka, I., Y. Tsutsumi-Ishii, S. Yomogida, and T. Yamashita. 1997. Isolation of cDNA encoding guinea pig neutrophil cationic antibacterial polypeptide of 11 kDa (CAP11) and evaluation of CAP11 mRNA expression during neutrophil maturation. *J. Biol. Chem. 272:22742.*
22. Kirikae, T., M. Hirata, H. Yamasu, F. Kirikae, H. Tamura, F. Kayama, K. Nakatsuka, T. Yokochi, and M. Nakano. 1998. Protective effects of a human 18-killodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. *Infect. Immun. 66:1861.*
23. Sawa, T., K. Kurahashi, M. Ohara, M. A. Gropper, V. Doshi, J. W. Larrick, and J. . P. Wiener-Kronish. 1998. Evaluation of antimicrobial and lipopolysaccharide-neutralizing effects of a synthetic CAP18 fragment against *Pseudomonas aeruginosa* in mouse model. *Infect. Immun. 42:3269.*
24. Someya, A., H. Nunoi, T. Hasebe, and I. Nagaoka. 1999. Phosphorylation of p40-phox during activation of neutrophil NADPH oxidase. *J. Leukocyte Biol. 66:851.*
25. Le Roy, D., F. Di Padova, R. Tees, S. Lengacher, R. Landmann, M. . P. Glauser, T. Calandra, and D. Heumann. 1999. Monoclonal antibodies to murine lipopolysaccharide (LPS)-binding protein (LBP) protect mice from lethal endotoxemia by blocking either the binding of LPS to LBP or the presentation of LPS/LBP complexes to CD14. *J. Immunol. 162:7454.*

26. Adachi, Y., C. Satokawa, N. Ohno, H. Tamura, and T. Yadomae. 1999. Inhibition by a CD14 monoclonal antibody of lipopolysaccharide binding to murine macrophages. *J. Endotoxin Res. 5:139.*
27. Juan, T. S.-C., E. Hailman, M. J. Kelley, L. A. Busse, E. Davy, C. J. Empig, L. O. Narhi, S. D. Wright, and H. S. Lichenstein. 1995. Identification of a lipopolysaccharide binding domain in CD14 between amino acids 57 and 64. *J. Biol. Chem. 270:5219.*
28. Pennica, D., J. S. Hayflick, T. S. Bringman, M. A. Palladino, and D. V. Goeddel. 1985. Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. *Proc. Natl. Acad. Sci. USA 82:6060.*
29. Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol. 3:787.*
30. Tobias, P. S., K. Soldau, and R. J. Ulevitch. 1989. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J. Biol. Chem. 264:10867.*
31. Galanos, C., M. A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization of the lethal effects of endotoxin. *Proc. Natl. Acad. Sci. USA 76: 5939.*
32. Seydel, U., A. B. Schromm, R. Blunck, and K. Brandenburg. 2000. Chemical structure, molecular conformation, and bioactivity of endotoxins. *Chem. Immunol. 74:5.*
33. Shenep, J. L., R. P. Barton, and K. A. Mogan. 1985. Role of antibiotic class in the rate of liberation of endotoxin during therapy for experimental gram-negative bacterial sepsis. *J. Infect. Dis. 151:1012.*
34. Kirikae, T., M. Nakano, and D. C. Morrison. 1997. Antibiotic-induced endotoxin release from bacteria and its clinical significance. *Microbiol. Immunol. 41:285.*
35. Di Padova, F. E., H. Brade, G. R. Barclay, I. R. Poxton, E. Liehl, E. Schuetze, H. P. Kocher, G. Ramsay, M. H. Schreier, D. B. McClelland, et al. 1993. A broad cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. *Infect. Immun. 61:3863.*
36. Pollack, M., A. M. Espinoza, G. Guelde, N. L. Koles, L. M. Wahl, and C. A. Ohl. 1995. Lipopolysaccharide (LPS)-specific monoclonal antibodies regulate LPS uptake and LPS-induced tumor necrosis factor-α responses by human monocytes. *J. Infect. Dis. 172:794.*
37. Nassif, X., J. C. Mathison, E. Wolfson, J. A. Koziol, R. J. Ulevitch, and M. So. 1992. Tumor necrosis factor α antibody protects against lethal meningococcaemia. *Mol. Microbiol. 6:591.*
38. Scott, M. G., A. C. E. Vreugdenhil, W. A. Buurman, R. E. W. Hancock, and M. R. Gold. 2000. Cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J. Immunol. 164:549.*
39. Hancock, R. E. W., and M. G. Scott. 2000. The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. USA 97:8856.*
40. Iwagaki, A., M. Porro, and M. Pollack. 2000. Influence of synthetic antiendotoxin peptides on lipopolysaccharide (LPS) recognition and LPS-induced proinflammatory cytokine responses by cells expressing membrane-bound CD14. *Infect. Immun. 68:1655.*
41. Dankesreiter, S., A. Hoess, J. Schneider-Mergener, H. Wagner, and T. Miethke. 2000. Synthetic endotoxin-binding peptides block endotoxin-triggered TNF-α production by macrophages in vitro and in vivo and prevent endotoxin-mediated toxic shock. *J. Immunol. 164:4804.*
42. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443.*
43. Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol. 12:20.*
44. Hoess, A., S. Watson, G. R. Siber, and R. Liddington. Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1:5 Å resolution. *EMBO J. 12: 3351.*
45. Stelter, F. 2000. Structure/function relationships of CD14. *Chem. Immunol. 74: 25.*
46. Sano, H., H. Sohma, T. Muta, S. Nomura, D. R. Voelker, and Y. Kuroki. 1999. Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14. *J. Immunol. 163:387.*
47. Matsuura, K., M. Setoguchi, N. Nasu, Y. Higuchi, S. Yoshida, S. Akizuki, and S. Yamamoto. 1989. Nucleotide and amino acid sequences of the mouse CD14 gene. *Nucleic Acids Res. 17:2132.*
48. Nagaoka, I., S. Hirota, S. Yomogida, A. Ohwada, and M. Hirata. 2000. Synergistic actions of antibacterial neutrophil defensins and cathelicidins. *Inflamm. Res. 49:73.*